*Acta Medica et Biologica*
*Vol. 47, No.4, 139-146, 1999*

# Rat 3Y1 Cell Retains Suppressive Activity for HTLV-I TAX-mediated Transformation

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*Received January 25 1999; accepted October 29 1999*

**Summary. Tax is an own transcriptional activator of human T-cell leukemia virus type I (HTLV-I) virus which is a causative agent of adult T-cell leukemia (ATL). Expression of the Tax protein in the rodent fibrolast, Rat-1 cells, leads to an anchorage independent growth in soft agar and tumor formation in athymic mice. Rat 3Y1 cells, originating from Fisher Rat fibroblasts, are known to be a permanent cell line exhibiting a normal phenotype and have been used for assays of transformation by several oncogenes. Although Tax was able to *trans*-activate satisfactorily the HTLV-I long terminal repeat (LTR) and NF-κB dependent promoter in 3Y1 cells, the cells expressing Tax could neither form visible colonies in soft agar nor elicit tumors in nude mice. Cell fusion experiments suggested that 3Y1 cells might have some dominant suppressive activities for Tax-mediated transformation. Since the constitutive activation of the NF-κB pathway is suggested to be responsible for Tax-mediated transformation of rat fibroblasts, 3Y1 cells might be useful materials to obtain suppressor candidates related to the NF-κB family involved in oncogenic properties or to elucidate other unknown pathways for Tax-mediated transformation of the rat fibroblast. At least p53 mutation is not involved in cooperation with interaction of *tax* gene for transformation.**

**Key words**—Rat 3Y1, Tax, transformation, CREB/ATF, NF-κB.

## INTRODUCTION

Human T-cell leukemia virus type I (HTLV-I) is a causative agent of adult T-cell leukemia (ATL)[1]. HTLV-I has a unique *pX* region between *env* and 3'long terminal repeat (LTR) in addition to common structures of retroviral genomes[2]. This *pX* region encodes three viral proteins: Tax, Rex, and p21[3-5]. Tax is known to be a transcriptional *trans*-activator of the HTLV-I LTR[6] and it also *trans*-activates other viral and cellular promoters[7-10] including the cyclic adenosine monophosphate response element binding protein/activating transcription factor (CREB/ATF) pathway and the NF-κB/Rel pathway.

Previous studies have proven the oncogenic potential of Tax using fibroblasts. Coexpression of the *tax* gene with the activated *ras* gene renders primary rat fibroblasts tumorigenic[11]. The expression of Tax transforms established rodent fibroblasts, Rat-1 cells[12]. It is required for maintenance of the transformed phenotype, and the stability of Tax protein is correlated with its transforming ability[13,14]. Recent studies have shown that the constitutive activation of NF-κB is essential for a Tax-mediated transformation of Rat-1 cells[15].

Although viral infection occurs in most cases at infancy, ATL shows low incidence rates and a long latency period[16]. Aberrant chromosomal abnormalities found in ATL leukemic cells and the long latency of ATL implies a multistep mechanism of leukemogenesis including the loss of tumor suppressor genes functions. On the assumption that the long latency of ATL is based upon the loss of suppressive genes, we became interested in suppressive factors (genes). If we could obtains the rodent fibroblastic cell lines which are resistant for Tax-mediated transformation, they should be valuable materials to search for suppressive factors by means of comparing resistant with sensitive cells for Tax-mediated transformation. Since the constitutive activation of NF-κB activation is essential for the transformation of Rat-1 cells, such cells also might be useful materials to pick up suppressive factors for NF-κB-

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related transformation. A series of transformation experiments provided us with another rat fibroblastic cell line as a candidate for this purpose, the 3Y1 cell.

Rat 3Y1 cells[17], as well as Rat-1 cells, are untransformed fibroblastic cells derived from embryos of the Fisher strain rat[18]. 3Y1 cells have been used in transformation experiments with many viral agents. In respect to anchorage independency among the phenotypes associated with transformation, 3Y1 cells are sensitive to the mouse polyoma virus, adenovirus type 12, Rous sarcoma virus, *v-H-ras* oncogene and N-methyl-N-nitro-N-nitrosoguanidine treatment, but not to simian virus 40 or the E1A region of adenovirus type 12[19].

In this report we demonstrated that 3Y1 cells contrasted with Rat-1 cells in not being susceptible to Tax-mediated transformation when evaluated both by colony formation assays in soft agar and by tumorigenicity in athymic mice. To investigate the differences between the two cell lines in their susceptibility to Tax-mediated transformation, we performed fusion experiments between Rat-1 and 3Y1 -derived cell lines expressing Tax to see whether resistant cells retain suppressive factors or not.

Since 3Y1 cells have been suggested to retain a suppressor activity for Tax-mediated transformation, we first became interested in the mutation of the p53 gene as a suppressor candidate, because the mutation of the p53 gene has been reported to be involved in a number of human cancers[20] including some cases of ATL[21-23]. We investigated the effect of mutant p53 regarding its cooperation in the interaction of the *Tax* gene in 3Y1 cells.

We here propose the possible existence of a suppressive gene(s) in 3Y1 cells which are resistant for Tax-mediated transformation with constitutive activation of NF-$\kappa$B pathway.

## MATERIALS AND METHODS

### Cells, Plasmids, Transfection

The rat fibroblastic cell line 3Y1 was presented by G. Kimura. The *v-src* (SR)-, *v-H-ras* (HR)- and SV40 (SV)-transformed 3Y1 cell lines {established by G. Kimura[24,25]} were obtained from Japanese Cancer Research Resources Bank. Cells were routinely cultivated as described[13]. For the establishment of cell lines expressing Tax, Rat-1 cells were transfected with pSV2bsr[26] and pKCR40M[12] by the calcium phosphate precipitation method, followed by glycerol shock at 4 hour after transfection.

One day after transfection, cells were cultured in a selection medium containing 10 $\mu$g/ml of blasticidin S hydrochloride (Funakoshi). After 7 to 10 days, blasticidin-resistent colonies were isolated from cultures transfected with pKCR40M. Cells transfected with pSV2bsr and salmon sperm DNA were collected to give a polyclonal cell population as a control (RSmix).

3Y1 cells were transfected with pH2R40M or pH2Rneo[12]. After cultivation in a selection medium containing 600 $\mu$g/ml of G418 (Gibco), several clones were selected from cultures transfected with pH2R40M. Cells transfected with pH2Rneo were selected in the same way, pooled, and designated as YNmix (control).

Human mutant p53 expression vectors, which have a CMV promoter, were received from Dr. Bert Vogelstein[27,28].This experiment used two kinds of mutant p53 genes, p53 <175> and p53 <273>, which have a point mutation at each nucleotide number. First, we constructed pKCRbsrtax replacing the *neo* gene of pH2R40M[12] by the Pvu2-Pstl fragment of pSV2bsr[26]. After the introduction of pKCRbsrtax into 3Y1 cells and the selection with blasticidin, some clones were chosen (bsrtaxcl. 1 and bsrtaxcl. 2) and many drug resistant clones were mixed (bsrtaxmix). Cells transfected with pSV2bsr were cultured in the same way and collected (bsrmix). Next, human mutant p53 expression vectors or a control vector carrying no insert of the p53 coding region (CMV) were introduced into the bsrmix or bsrtaxmix. After selection in G418, some clones were chosen and many clones were mixed.

### Cell fusion and colony-forming assay

One day before cell fusion, cells (approximately $5\times10^5$ cells each) were plated in 2-cm dishes and co-cultured overnight. After being washed once with PBS, cells were treated with a solution of serum-free Dulbecco's modified Eagle's medium (DMEM) containing 50% polyethylene glycol 1500 (BDH) for 2 min, washed twice with PBS, and cultured in DMEM containing 10% fetal calf serum overnight. The next day, cells were trypsinized and replated onto two 10 -cm dishes in the presence of both G418 (600 $\mu$g/ml) and blasticidin (5 $\mu$g/ml). Cells were refed every 3 to 4 days with the selection medium for 7 days and collected to make polyclonal populations.

Polyclonal and clonal cell lines (approximately $5\times10^3$ cells/dish) were suspended in a complete medium containing 0.33% low melting agar (FMC) for three weeks in duplicates. Colonies over 60 $\mu$m in diameter were counted and colony-forming efficiencies were calculated.

### Western blotting

For immunoblotting, cell lysates were prepared from 80% confluent cultures with a lysis buffer containing 50mM Tris-HCl (pH7.5), 150mM NaCl, 4mM EDTA, 1% NonidetP-40 (NP-40) and 2mM phenylmethylsulfonyl fluoride (PMSF) for the Tax protein; and Tris-HCl (pH8.0), 150mM NaCl, 1% NP-40, 4mM EDTA, 0.1% SDS, 0.5% deoxycholate, 0.01% PMSF, 1 mM sodium orthovanadate, 5 mM sodium fluoride, aprotinin (1$\mu$g/ml) and leupeptin (1$\mu$g/ml) for the p53 protein. Protein concentrations were determined by the Bio-Rad protein assay. Samples (100$\mu$g) were subjected to electrophoresis on 10% polyacrilamide gels, followed by the transfer to Immobilon-P (Millipore). Membranes were probed with an anti-Tax monoclonal antibody MI73[29] and $^{125}$I-labelled protein A (Amersham) or anti-p53 mAb (Ab-2; Oncogene Science) and anti-mouse IgG alkariphosphatase-conjugate antibody (Promega).

### Chloramphenicol acetyl transferase (CAT) assay

CAT assays were performed as described previously[13]. PHLCl[29] contains the HTLV-ILTR linked to the bacterial CAT gene. Ig$\kappa$tkCAT is a NF-$\kappa$B-dependent reporter construct[15]. pCMV$\beta$[30] was co-transfected as an internal control. Transfection efficiencies were normalized by $\beta$-galactosidase activity. Conversion to acetylated [$^{14}$C] chloramphenicol was quantitated by a BAS 2000 analyzer (Fuji, Tokyo).

**Table 1.** Anchorage independency of cell lines and hybrid cells

| Cells | Colony forming efficiency | | Mean diameter ($\mu$m) |
|---|---|---|---|
| | Experimental no. 1 | 2 (%) | |
| RSmix | 0 | 0 | |
| RbT-5 | 90.0 | 94.8 | 166,138 |
| YNmix | 0 | 0 | |
| YT-1A | <1 | <1 | |
| YT-2A | 0 | 0 | |
| SV | 0 | | |
| HR | 13.2 | | 107 |
| SR | >100 | | 249 |
| RSmix x YNmix | 0 | 0 | |
| RSmix xYT-1A | 0 | | |
| RSmix xYT-2A | 0 | | |
| YNmix x RbT-5 | 4.4 | 2.7 | 156,138 |
| RbT-5 x Rmix | 55.9 | 49.5 | 121,180 |

Summary of colony forming efficiencies of Rat-1, 3Y1 and hybrid cells. Colonies over 60 $\mu$m in diameter were counted, and colony forming efficiencies were calculated. RSmix, control of Rat-1 cell; RbT-5, clone of Rat-1 cells expressing Tax; YNmix, control of 3Y1 cell; YT-1A and YT-2A, clones of 3Y1 cells expressing Tax. SV, HR and SR refer to 3Y1 cells as described in Materials and Methods.

## RESULTS

### Rat-1 cells and 3Y1 cells expressing Tax

We established a clonal cell line, RbT-5, transfecting Rat-1 cells with pKCR40M and pSV2bsr. This cell line had the characteristics of Tax-transformed Rat-1 cells reported previously[12,13] (Table 1). They exhibited small sized cellular bodies, accumulated on a monolayer culture, and formed colonies in soft agar. RSmix in a pooled population transfected with pSV2bsr and salmon sperm DNA.

YT-1A and YT-2A are clonal cell lines derived from 3Y1 cells transfected with pH2R40M[12]. These two clones, expressing different amounts of Tax (Fig. 1, lanes 4 and 5), had more sharp, spindle-shaped bodies and were a little smaller in size compared with the parental 3Y1 cells[17,19]. Both these clones as well as the mixed population of 3Y1 expressing Tax were unable to form visible colonies in 0.33% soft agar, suggesting that 3Y1 cells are resistant to Tax-mediated transformation (Fig. 2). In addition, YT-2A could not elicit tumors in nude mice (data not shown). As reported previously[19], the *v-src*-and *v-H-ras*-transformed 3Y1 cells (SR and HR) formed colonies efficiently, while SV40-transformed ones (SV) did not (Fig. 2, Table 1 and data not shown).

### Fusion experiments

Rat-1 cells carrying the *neo* gene and 3Y1 cells carrying the *bsr* gene were fused. After cultivation in the selection medium containing both G418 and blasticidin for 7 days, drug-resistant cells were collected and subjected to a soft agar assay. For control of the fusion effect, RbT-5 was fused with Rat-1 cells {Rmix[13]} which was a pooled population transfected

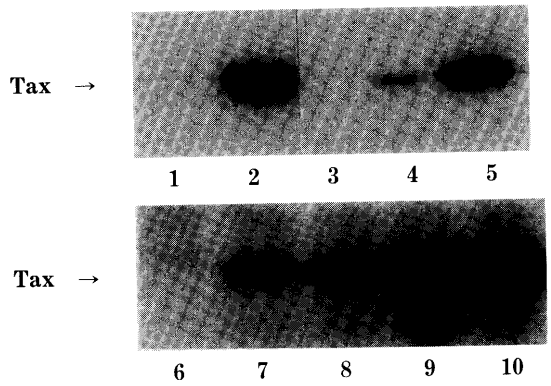



**Fig. 1.** Western blot analysis of the Tax protein expressed in Rat-1 cells, 3Y1 cells and hybrid cells. Tax was detected by immunoblot techniques using the Tax-specific-monoclonal antibody (MI73) and $^{125}$I-labeled protein A. Only one band for Tax is visible. Arrow indicates bands for Tax. lane 1, RSmix (Rat-1); lane 2, RbT-5 (Rat-1, Tax); lane 3, YNmix (3Y1); lane 4, YT-1A (3Y1, Tax); lane 5, YT-2A (3Y1, Tax); lane 6, hybrid cells between RSmix (Rat-1) and YNmix (3Y1); lane 7, hybrid cells between RSmix (Rat-1) and YT-1A (3Y1, Tax); lane 8, hybrid cells between RSmix (Rat-1) and YT-2A (3Y1, Tax); lane 9, hybrid cells between YNmix (3Y1) and RbT-5 (Rat-1, Tax); lane 10, hybrid cells between RbT-5 (Rat-1, Tax) and Rmix (Rat-1).

with pH2Rneo. Hybrid cells produced by the fusion of Rmix with RbT-5 exhibited a mild reduction of colony forming efficiency (Table 1, RbT-5x Rmix). In contrast, hybrid cells between YNmix and RbT-5 showed an evident reduction suggesting some suppressive mechanisms for Tax-mediated transformation in 3Y1 cells (Table 1, YNmix x RbT-5). To confirm the Tax expression in hybrid cells, we performed Western blot analysis. While YT-2A and RbT-5 expressed similar levels of Tax protein, YT-1A had different levels based on their clonic characteristics (Fig. 1, lanes 1-5). Hybrid cells thus expressed sufficient levels of Tax protein (Fig. 1, lane 6-10).

### Chrolamphenicol acetyl transferase (CAT) assay

It has been reported that Tax modulates the transcription of viral and cellular genes through interactions with diverse cellular factors, including CREB/ATF and NF-$\kappa$B/Rel pathways[31]. We investigated whether Tax could activate these two pathways by CAT assays using reporter plasmids pHLC1[13] and Ig$\kappa$tkCAT[15] (see Materials and Methods). Fig. 3 shows that Tax can satisfactorily activate both pathways in 3Y1 cells. When the Ig$\kappa$tkCAT reporter plasmid was transfected into YT-2A expressing Tax

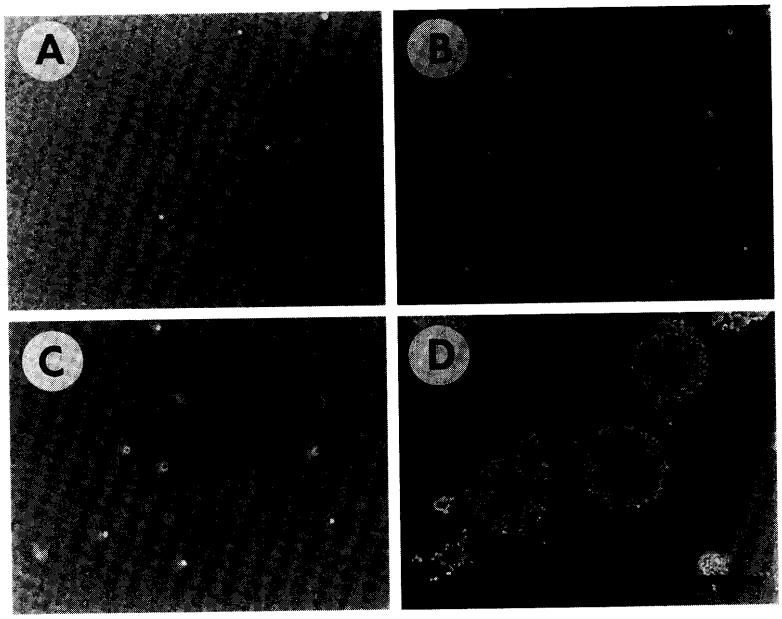
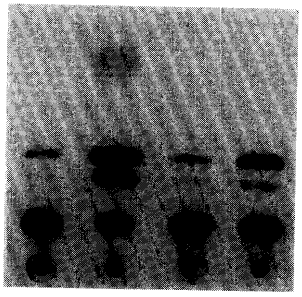



**Fig. 2.** Photographs of colony formation of 3Y1 cells in 0.33% soft agar. Cells were prepared as described in Materials and Methods. After 3 weeks, cells were photographed microscopically. *Bar* indicates 100 $\mu$m. **A.** YNmix (3Y1); **B.** YT-1A (3Y1, Tax); **C.** YT-2A (3Y1, Tax); **D.** v-src-transformed 3Y1 cells.



| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| pHLC1 | + | + | | |
| lgltkCAT | | | + | + |
| pKCRH2 | + | | + | |
| pKCR40M | | + | | + |
| % conversion | 4.0 | 82.4 | 3.8 | 25.7 |

**Fig. 3.** CAT assay. HTLV-1 LTR-dependent (pHLC1) or NF-$\varkappa$B-dependent (Ig$\varkappa$tkCAT) reporter constructs were introduced into 3Y1 cells together with either pKCRH2 (control) or pKCR40M (Tax expression constructs)[15]. Transfection efficiencies were normalized by an internal control, CMV$\beta$-gal. CAT assay was performed as described in Materials and Methods.

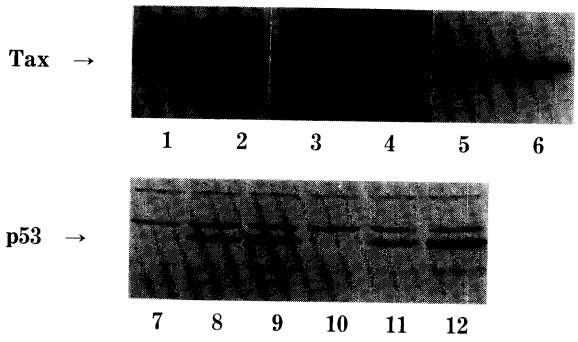



**Fig. 4.** Western blot analysis of Tax and p53 proteins. Lanes 1 and 7, bsr-CMV (3Y1); lanes 2 and 8, bsr-p53 <175> (3Y1, mutant p53); lenes 3 and 9, bsr-p53<273> (3Y1, mutant p53); lanes 4 and 10, bsrtax-CMV (3Y1, Tax); lanes 5 and 11, bsrtax-p53 <175> (3Y1, Tax, mutant p53); lanes 6 and 12, bsrtax-p53 <273> (3Y1, Tax, mutant p53). Each protein was detected as described in Materials and Methods. To detect the Tax protein, an anti-mouse IgG alkariphosphatase-conjugate antibody was used.

steadily, CAT activity was about 13-fold higher compared wiht those with control plasmids (data not shown).

**Table 2.** Anchorage independency of 3Y1 cell expressing Tax and human mutant p53

| 3Y1 cell | Colony forming efficiency (%) | Mean diameter ($\mu$m) |
|---|---|---|
| bsr mix | 0 | |
| bsrtax mix | <1 | |
| bstax cl.1 | 1.9 | 76 |
| bstax cl.2 | 0 | |
| bsr-CMV | 0 | |
| bsr-p53 <175> | 0 | |
| bsr-p53 <273> | 0 | |
| bsrtax-CMV | 1.1 | 72 |
| bsrtax-p53 <175> | 4.2 | 77 |
| bsrtax-p53 <273> | <1 | |

Colony forming efficiencies of 3Y1 cells. Cells were grown in soft agar for 3 weeks and colonies over 60 $\mu$m were evaluated. bsr mix, control of 3Y1 cell; bsrtax mix, 3Y1 cells expressing Tax; bsrtax cl.1 and bsrtax cl.2, clones of 3Y1 cells expressing Tax; bsr-CMV, control of 3Y1 cells; bsr-p53 <175> and bsr-p53 <273>, 3Y1 cells expressing mutant p53; bsrtax-CMV, 3Y1 cells expressing Tax; bsrtax-p53 <175> and bsrtax-p53 <273>, 3Y1 cells expressing both Tax and mutant p53.

### Involvement of mutant p53 gene

We introduced the human mutant p53 gene together with the Tax expression vector into 3Y1 cells. We used human mutant p53 to detect the exogenous mutant p53. For this purpose, we established the Rat-1 cell expressing Tax again using pKCRbsrtax which has the *bsr* gene *in cis*. Fig. 4 shows the expression of both Tax and mutant p53. The results of colony formation efficiencies are summarized in Table 2. The human mutant p53 did not show any positive effects (Table 2, bsr-p53 <175>, bsr-p53 <273>). However, some clones expressing Tax exhibited small effects forming small colonies microscopically at a very low percentage (bsrtaxcl. 1, bsrtax-CMV, bsrtax-p53 <175>). We could not observe visible colonies macroscopically on any 3Y1 cells expressing both Tax and p53.

## DISCUSSION

The finding that the *pX* sequence is detectable even in the late stages of leukemic lymphocytes[32] leads to the assumption that a multistep mechanism including the activation of the oncogenes and loss of suppressor

genes might cause the onset of ATL. Comparison of sensitive and resistant cell lines for Tax-mediated transformation might provide valuable clues to obtain the suppressive factors by molecular biological methods, e.g. subtraction or differential display[33], which could make it easier to isolate genes responsible for the suppressive function. Although fibroblasts differ from lymphoid cells in their growth regulation, the isolation of suppressor genes in the fibroblast system would be helpful in analyzing the mechanism of the onset and long period of latency of ATL. From a series of experiments, we found that 3Y1 cells could be instrumental candidates for this purpose; these are rodent fibroblastic cells, similar to Rat-1 cells with respect to their origin, and have been used for transformation experiments providing useful information. Although Rat-1 cells expressing Tax could form visible colonies in soft agar indicating oncogenic potential, 3Y1 cells with their sufficient expression level of Tax protein could not, indicating that 3Y1 cells are resistant to Tax-mediated transformation. Although the bsrtaxmix, bsrtaxcl. 1 and bsrtax-CMV (Table 2), 3Y1 cells expressing Tax, exhibited some small sized colonies at a very low percentage, these colonies were not visible macroscopically and growth ceased after some limited proliferation in soft agar. It is clear that this small increased growth property is different from the positive colony formation indicating oncogenic potential. Also, we confirmed that 3Y1 cells exprssing Tax did not produce tumors on athymic nude mice (data not shown). We next analyzed whether 3Y1 cells contain cellular factors which suppress the transformation by Tax or merely lack factors required for transformation. Cell fusion experiments have so far provided valuable information on such a question[34,35].Since hybrid cells are known to lose chromosomes after varying intervals of time[35], we evaluated them as pooled populations at early passages with the same process avoiding the influence of a time course. Hybrid cells produced by the fusion of Rmix (Rat-1 cells) with RbT-5 (Rat-1 cells expressing Tax) showed a mild reduction in colony forming efficiencies (Table 1, RbT-5 x Rmix). We speculate that there might be very unstable hybrid cells which could not grow in the medium through these early passages. In contrast, although hybrid cells between YNmix (3Y1 cells) and RbT-5 (Rat-1 cells expressing Tax) had a sufficient level of Tax protein (Fig. 1, lane 9), these cells showed an evident reduction in colony forming efficiencies, suggesting some suppressive factors against Tax-mediated transformation in 3Y1 cells (Table 1, YNmix x RbT-5), although these mechanisms are not known.

Recently Inoue et al. have reported that normal human fibroblasts contain suppressor gene(s) which can block Tax-mediated transformation by fusion experiments[34]. Among the several suppressive genes reported so far, the representative human suppressor gene involved in cancers is p53. The p53 gene mutation has been shown to be involved in the progression of various human cancers[20,36],and some reports have suggested the participation of a mutation of p53 gene in ATL, especially in the acute form[21-23].We therefore investigated the effect of mutant p53, which has been shown to have a dominant negative effect biologically. We introduced the human mutant p53 gene together with the Tax expression construct into 3Y1 cells. After confirming the exprssions of each protein, we investigated the oncogenic potentials by soft agar assay, resulting in no colony formation. We thus speculated that the cooperation of these genes might be insufficient or additional factors are indispensable for the transformation of 3Y1 cells.

It has been reported that Tax modulates the transcription of viral and cellular genes through interactions with diverse cellular factors, including CREB/ATF and NF-$\kappa$B/Rel, mediated through distinct regions of Tax[31,37]. Recent reports have suggested that the constitutive activation of NF-$\kappa$B is essential for a Tax-mediated transformation of rat fibroblasts, demonstrating that the stable co-expression of the NFKB2 precursor, known as a member of the I$\kappa$B proteins, with wild-type Tax blocked transformation as well as eliminated aberrant NF-$\kappa$B activation by Tax without interference with the HTLV-I LTR-mediated *trans*-activation[15]. Therefore we investigated Tax activation of the NF-$\kappa$B pathway in 3Y1 cells by CAT assays using a $\kappa$B-dependent CAT reporter construct. Cells were transiently co-transfected with a Tax expression construct and CAT reporter plasmids containing the HTLV-I LTR or an NF-$\kappa$B-dependent promoter. As shown in Fig. 3, Tax can satisfactorily activate transcription from both of the pathways in 3Y1 cells. Since the *trans*-activation of Tax observed in transient expression experiments do not necessarily assure us of the constitutive transcriptional activation of the target genes in 3Y1 cells steadily expressing Tax, we transfected the NF-$\kappa$B-dependent reporter construct into YT-2A expressing Tax steadily to investigate the constitutive activation of the NF-$\kappa$B/Rel pathway. The result showed intense activation (more than 10 -fold) of the NF-$\kappa$B/Rel pathway by Tax in 3Y1 cells (data not shown). It is of interest that 3Y1 cells are resistant for Tax-mediated transformation with the constitutive activation of NF-$\kappa$B pathways. This result might suggest unknown pathways which play a

central role for the Tax-mediated transformation of rat fibroblasts in addition to NF-κB/Rel pathways. Another possibility is that 3Y1 cells might retain the suppressive factors (genes) which could block the transformation caused by the gene family governed by NF-κB/Rel pathways. Since oncogenic potentials have been reported for several Rel/NF-κB family members[38,39], an attempt to obtain some suppressor candidates might be valuable for understanding how NF-κB regulates complicated cell growth-related genes.

In conclusion, 3Y1 cells can be used to elucidate the mechanism not only for Tax mediated-transformation, but also for the gene regulation of NF-κB families. We anticipate that a comparison between Rat-1 cells and 3Y1 cells will provide new insights into the molecular mechanism of gene regulation related to Tax.

**Acknowledgments.** These works were performed at the Institute for Virus Research, Kyoto University. We thank Dr. Makoto Uchiyama for his generous support and critical reading of the manuscript. We also thank Ms. Michiko Simizu and Ms. Yuko Soga for preparing the figures.

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